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On the reactivity and selectivity of donor glycosides in glycochemistry and glycobiology: trapped covalent intermediates

Marthe T. C. Walvoort, Gijsbert A. van der Marel, Herman S. Overkleeft* and Jeroen D. C. Codée*

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The reactivity of sugar donors and the stability of covalent intermediates formed in both chemical and biological systems is an active subject of study in both glycochemistry and glycobiology. Knowledge of the structure of these intermediates is vital for understanding reactivity and stereoselectivity in glycosidic bond formation, and in glycosidic bond destruction in the case of enzymatic hydrolysis. For chemical reactions, tuning of the electron-withdrawing power of the carbohydrate side chains allows for stabilization of covalent anomeric triflates thereby enabling chemo-, regio- and stereoselective glycosylations. Retaining glycosidase-mediated hydrolysis reactions in turn often involve a covalent intermediate. The existence of such covalent intermediates was convincingly demonstrated at the beginning of this century by making use of modified glycosyl substrates tuned such that stable adducts are formed efficiently but the ensuing hydrolysis is slowed down. Recently this concept has also been used in the design of glycosidase activity-based probes. This review describes recent investigations on different carbohydrate decoration patterns to influence both chemical and biological reactivity and selectivity.

Introduction

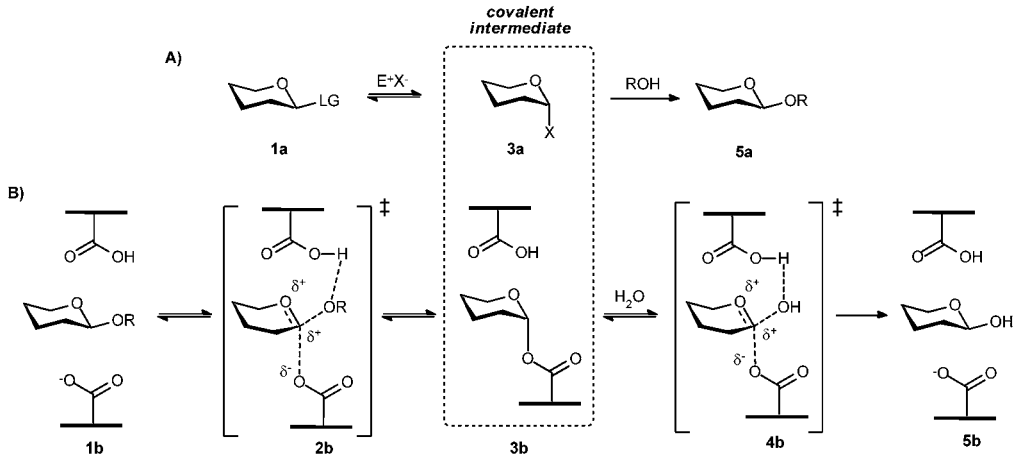
Amongst the most fundamental processes in glycochemistry and glycobiology are the union of two carbohydrate building blocks in a glycosylation reaction, and the breaking of a glycosidic bond in the hydrolysis of a glycoconjugate by the action of a glycoside hydrolase. Numerous mechanistic pathways can lead to a glycosylation event. This holds true for both chemical glycosylation reactions and enzymatic hydrolysis of glycosidic bonds. When looking in close detail, it becomes apparent that these processes share some common mechanistic features. Detailed analysis of both the chemical glycosylation and the glycosidase-mediated hydrolysis of a glycosidic bond can assist in the development of efficient stereoselective glycosylation reactions, in guiding the design of tailored probes to study glycosidases, and in the development of potent and selective inhibitors of these enzymes.

As depicted in Scheme 1A, a chemical glycosylation reaction starts with the activation of an anomeric leaving group in donor **1a** by a promoter (E^+X^-), followed by expulsion of the aglycone. The transient oxocarbenium ion can be intercepted by the counter ion of the promoter (X^-) to form covalent intermediate **3a**. Attack of the nucleophile, either on the covalent intermediate or the oxocarbenium ion (not shown in the scheme), results in the formation of the glycosidic bond, as in **5a**.

Analogously, when a glycoside enters the active site of a (retaining) glycoside hydrolase (**1b**, the so-called 'Michaelis complex', Scheme 1B), a general acid/base residue protonates the leaving group while a nucleophilic residue attacks the anomeric center (as in transition state **2b**). A covalent linkage is formed between the glycoside and the enzyme (**3b**) with inversion of configuration at the anomeric center of the glycoside. Subsequently, this species can be attacked by water from the opposite face to release the glycoside (**4b**) and produce hemiacetal product **5b**, with overall retention of configuration. Knowledge on the nature of the covalent intermediates **3a** and **3b** provides fundamental insight into the mechanistic pathways that are in operation during the course of a chemical glycosylation or enzymatic hydrolysis reaction. The use of modern spectroscopic techniques in combination with cleverly designed 'substrates' has led to a deep insight into the said reaction mechanisms.

In this Perspective some studies on these common mechanistic features in glycobiology and glycochemistry are highlighted, with a focus on lessons learned with respect to similarities in glycosylation events, such as they occur in a reaction vessel and in nature. Drawing on selected examples, it is laid out how electron-deprived carbohydrates can be of use to generate covalent intermediates, both in glycochemistry and

Leiden Institute of Chemistry, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. E-mail: h.s.overkleeft@lic.leidenuniv.nl; jcodee@chem.leidenuniv.nl



Scheme 1 Mechanisms of chemical glycosylation (A) and enzymatic hydrolysis by a retaining glycosidase (B).

glycobiology, and used to study mechanisms underlying chemical glycosylation reactions and enzymatic hydrolysis processes.

Glycobiology

The reactivity of a glycoside in a glycosylation or hydrolysis reaction is determined by its ability to accommodate the positive charge that develops at the anomeric center during expulsion of the activated aglycone. In glycochemistry, the influence of the substituents on the carbohydrate core is well established and the reactivity of a carbohydrate building block can be tuned through the use of different protecting groups. Also the orientation of the substituents on the ring is of influence, and studies on glycoside hydrolysis have revealed that the rate of the reaction is directly related to the number of axial hydroxyl substituents.[1] Presumably, an axially positioned hydroxyl function has a smaller deactivating effect on the developing positive charge in the transition state compared to an equatorially oriented hydroxyl. Also the position of the substituent on the carbohydrate core is of importance. The rate of hydrolysis of a series of *x*-deoxy-*x*-fluoro- and *x*-deoxy-dinitrophenylglucosides (with *x* indicating the position of the hydroxyl on the pyranosyl core that is substituted with a fluoride or hydrogen, respectively) was investigated, and in the fluoro series, the order of reactivity was revealed to be 2-fluoro < 4-fluoro < 3-fluoro < 6-fluoro < parent sugar. This trend was reversed in the corresponding *x*-deoxyglucoside series.[2] These results were explained by the deactivating effect of the electron-withdrawing fluorine atom and the activating effect of the deoxy center, on the formation of the oxocarbenium ion-like intermediate.

Based on the deactivating effect of the C2-fluorine atom, Withers and colleagues designed the 2-deoxy-2-fluoroglycosides as mechanism-based enzyme inhibitors.[3] By introducing an electron-withdrawing fluorine atom next to the anomeric center of a glycoside, the hydrolysis of covalent glycosyl–enzyme adducts (**3b** → **5b**) is considerably tempered. To accelerate the glycosylation step (**1b** → **3b**), which is also retarded by the action of the fluorine atom, a potent anomeric leaving group was introduced, typically a fluoride, nitrophenyl or dinitrophenyl. Because the second step of the double displacement reaction sequence (**3b** → **5b**) is slowed down more than the first displacement event (**1b** → **3b**), exposing a glycosidase to these inhibitors results in accumulation of the covalent glycosyl–enzyme intermediate (**3b**). With these probes, the nucleophilic residues of many retaining β-glycoside hydrolases have been characterized by mass spectrometry after enzymatic digestion of the stable adducts **3b**. In most cases, the nucleophile of a glycoside hydrolase is the carboxylate moiety of an aspartic acid or glutamic acid residue,[4] but sialidases can also employ a tyrosine residue as the catalytic nucleophile.[5] Insightful information on the three-dimensional structure of inhibitor–glycosidase complexes has been obtained through X-ray crystallography studies on the inhibitor-bound enzymes. This has revealed that β-glucosidase[6] and most β-xylosidase enzymes[7,8] produce an α-linked glycosyl adduct with the pyranosyl chair adopting a $^4C_1$ conformation (**6**, Fig. 1).

Interestingly, in several β-mannosidases, the covalent α-mannosyl intermediate takes up an $^OS_2$ skew boat conformation (**7**, Fig. 1). The different conformations of the bound glucosides and mannosides provide an explanation why β-glucosidase and β-mannosidase enzymes display a high degree of similarity (practically all β-mannosidases belong to glycoside hydrolase families, which also contain β-glucosidases, see www.cazy.org),[9] particularly around the C2–OH position of the active site, while glucose and mannose are epimeric structures at C-2.[10] As depicted in Fig. 1, the covalent intermediates in the β-glucosidases and β-mannosidases place most ring

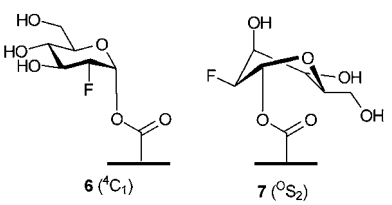


Fig. 1 Covalent intermediate **6** from β-glucosidase, and **7** from β-mannosidase.

substituents in a (pseudo-)equatorial position while positioning the anomeric substituent in a (pseudo-)axial orientation. These conformations are ideally suited to allow for nucleophilic displacement of the anomeric acyl group, and in the case of β-mannosidase, provide an explanation on how the enzyme manages to circumvent the steric hindrance by the C-2 substituent in the displacement event.

Deactivated glycosyl inhibitors have also been used to obtain structural information on the transient Michaelis complex (**1b**, Scheme 1). Davies and co-workers[11] were the first to report on a crystal structure of a Michaelis complex of an endo-β-1,4-glucanase enzyme (a member of the GH7 family) in complexation with a β-1,4-pentaglucoside substrate, featuring non-hydrolyzable sulfide linkages. This structure revealed that the proximal (−1) residue of the substrate was distorted away from the relaxed $^4C_1$ conformation. This finding was corroborated[12] by analysis of the crystal structures of a GH5 β-glucosidase, incubated with 2-deoxy-2-fluoroglucobioside **8** (Scheme 2) at pH 5.5, a pH at which the enzyme is inactive. In this Michaelis complex, the proximal (−1) residue takes up a $^1S_3$ skew boat conformation (**9**), placing the scissile C1–*O*-DNP linkage in a pseudo-axial position, ready for aglycone departure upon nucleophilic attack from the other side of the sugar ring.

Using probes **11**[13] and **12**,[14] the structures of the Michaelis complexes of mutant β-mannosidases of the GH2 and GH26 families were obtained. In these the proximal (−1) pyranosides appeared to adopt a $^1S_5$ skew boat conformation (**13**, Scheme 2). From these structures, the similarities between the β-mannosidases and β-glucosidases again become apparent. Both complexes place the substrate in a conformation that allows for the pseudo-axial displacement of the leaving group (the sugar or aglycone in the +1 position), while minimizing steric interactions of the incoming nucleophile with H-3 and H-5. *Ab initio* calculations show that the $^1S_5$ conformation of the mannose ring in the Michaelis complex of β-mannosidases best orchestrates the structural requirements for nucleophilic displacement, including bond elongation/shrinking, leaving-group orientation, and charge distribution.[15] Similarly, computation of the free-energy landscape (FEL) of β-glucose reveals that a structure approaching a $^1S_3/B_{3,O}$ conformation represents the optimal structure for displacement of a β-glucoside, as found in the Michaelis complexes described above.[10]

The conformations of the Michaelis complex and covalent intermediate together flank the transition state of the hydrolysis reaction (Scheme 1, **1b** and **3b**). Using Stoddart's hemisphere representation of pseudo-rotational itineraries, in which the accessible conformations of a pyranoside ring are depicted with the $^4C_1$ and $^1C_4$ chairs as the 'extremes' (Fig. 2),[16] the structure of the glycopyranosyl ring in the transition state can be deduced.

For the β-glucosidases described above, the $^1S_3$ Michaelis complex and the $^4C_1$ covalent adduct flank a $^4H_3$ half-chair conformation, implying this conformation for the glucopyranosyl oxocarbenium ion-like moiety in the transition state (**10**, Scheme 2 and Fig. 2, $^1S_5 \rightarrow {}^4H_3 \rightarrow {}^4C_1$).[17] Analogously, it can be deduced that the conformational itinerary for the hydrolysis of β-mannosides ($^1S_3 \rightarrow {}^OS_2$) passes through a $B_{2,5}$ boat conformation (**14**).[14,18] Notably, this boat structure, in which the anomeric center is partially sp^2-hybridized, resembles the conformation observed for D-mannono-1,5-lactone, also featuring an sp^2-hybridized anomeric center.[19] The occurrence of the $B_{2,5}$ conformation in the β-mannosidase transition state was further evidenced by the screening of a set of β-mannosidase inhibitors, where tight binding was observed with inhibitors having a boat (or similar) conformation.[20] Using

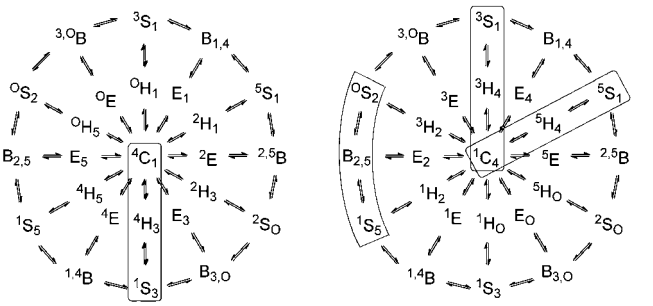
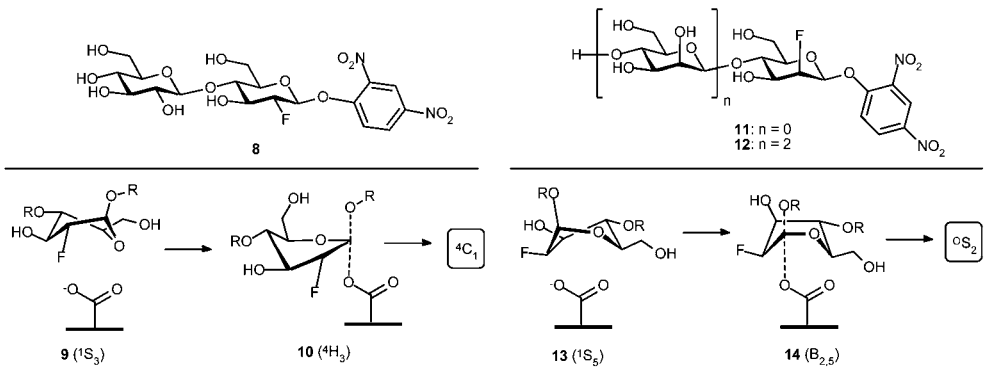


Fig. 2 Stoddart's hemisphere representations of pseudo-rotational itineraries. In boxes the itineraries described in this perspective.



Scheme 2 Left: probe **8**, and the conformational itinerary of β-glucosidases ($^1S_3 \rightarrow {}^4H_3 \rightarrow {}^4C_1$). Right: probes **11** and **12**, and the conformational itinerary of β-mannosidases ($^1S_5 \rightarrow B_{2,5} \rightarrow {}^OS_2$).

similar methods, the rotational itineraries of sialidases ($^6S_2 \rightarrow {}^6H_5 \rightarrow {}^2C_5$),[5] L-fucosidases ($^1C_4 \rightarrow {}^3H_4 \rightarrow {}^3S_1$),[21] and xylanases ($^1S_3 \rightarrow {}^4H_3 \rightarrow {}^4C_1$)[7a] have been deduced (Fig. 2).

In contrast to β-glycosidase inhibitors, such as **15** (Fig. 3), the 2-fluoro-α-glycosyl probes (**16**, Fig. 3) were found to be poor inhibitors of α-glycosidases. Kinetic studies have revealed that the C-2 fluorine substituent has a larger deactivating effect on the glycosylation step (**1b** → **3b**) of the α-linked probes than on the deglycosylating step (**3b** → **5b**), resulting in slow substrates instead of inhibitors.[22] It has been postulated that the hydrolysis of β-glycosides takes place with more positive charge development at the anomeric carbon atom in comparison to α-glycoside hydrolysis, which proceeds with the development of significant positive charge at the ring oxygen.[23] The deactivating effect of a C-2 fluorine thus has a greater impact on the mode of action of the β-glucosidase probes. The difference between α- and β-fused probes might also be explained by the intrinsic higher stability of the α-configured inhibitors, which benefit from the stabilizing anomeric effect.[24] As a result, the α-probes are less reactive in the glycosylation step (**1b** → **3b**). In addition, the covalent intermediate formed from α-glycosides has the higher energy β-configuration, and is easily hydrolyzed in the deglycosylation step (**3b** → **5b**). Taken together, these effects hamper the accumulation of the covalent glycosyl–enzyme adducts (**3b**). Also the relative importance of the presence of the C-2 hydroxyl can be at the basis of the observed differences between α- and β-glycosidases. For example, studies on human acid glucosylceramidase (GBA) revealed that deoxynojirimycin-based iminocyclitols lacking a C-2 hydroxyl (glucopyranose numbering) are weak inhibitors, much weaker than the corresponding 2-hydroxy derivatives, irrespective of the configuration of the C-3, C-4 and C-5 substituents. It may well be that the interaction within glycosidase active sites with a C-2 hydroxyl group through hydrogen bonding is a general and important feature. As an alternative to the 2-deoxy-2-fluoroglycosyl probes, 5-fluoroglycosides (**17**, Fig. 3) were designed as mechanism-based inhibitors for α-glycosidases.[25] With these, α-glucosidases,[26] α-mannosidases[27] and α-galactosidases[28] were covalently glycosylated, facilitating the characterization of the nucleophilic residues.

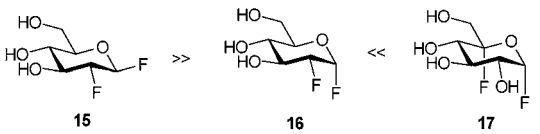
Although inverting glycosidases do not hydrolyze glycosides through the intermediacy of a covalent adduct, and therefore are beyond the scope of this Perspective, the GH47 α-mannosidase involved in *N*-glycan processing is worth mentioning because of the intriguing conformational changes taking place during the hydrolysis of the α-mannosidic linkage. Using non-hydrolyzable thiomannobioside **18** and known inhibitor 1-deoxymannojirimycin (**19**, Scheme 3), the crystal structures of both the Michaelis complex and the inhibitor–enzyme complex were obtained.[29] Interestingly, in the Michaelis complex the mannosyl residue at the −1 position (the "non-reducing end" mannoside) adopts a $^3S_1$ skew boat (**20**) to accommodate the anomeric substituent in a pseudo-axial orientation, and the mannoside takes up an unexpected $^1C_4$ chair in the product complex (**22**). These intermediates together flank a transition state in which the mannosyl cation adopts a $^3H_4$ oxocarbenium ion-like structure (**21**).

Recent studies from our laboratory have indicated that the β-stereoselectivity in glycosylations of mannuronic acid donors can be explained with a product-forming $^3H_4$ oxocarbenium ion-like transition state (*vide infra*). This is further endorsed by the observation that mannuronic acid lactone **23** having an sp^2-hybridized anomeric carbon, takes up a $^3H_4$ conformation,[30] in contrast to the $B_{2,5}$ conformation of D-mannono-1,5-lactone and the conformation of the mannosyl ring in the transition states in the β-mannosidases described above.

The covalent attachment of glycosyl inhibitors in the enzyme active site has been employed in activity-based protein profiling (ABPP). For this purpose, covalent inhibitors were converted into activity-based probes by grafting a fluorescent group or ligation handle to the carbohydrate ring to allow the visualization of the bound enzyme (Scheme 4). For instance, Vocadlo and Bertozzi used 2-fluoro-6-azidogalactosyl probe **24** to study β-galactosidase activity *in vitro*.[31] Overnight incubation of bacterial β-galactosidase LacZ with probe **24** was followed by a Staudinger ligation using a FLAG-phosphine. This allowed for Western blot analysis of the covalent glycosyl–enzyme adduct, after SDS-PAGE, using anti-FLAG-horseradish peroxidase (HRP). In a similar manner, 5-fluoro probe **25** was employed to inhibit *N*-acetyl-β-glucosaminidases, which could then be labeled with a phosphine-FLAG tag for Western blot analysis, or functionalized with an alkyne-functionalized biotin to allow for pull-down of the enzyme from a cell lysate using streptavidin resin.[32]

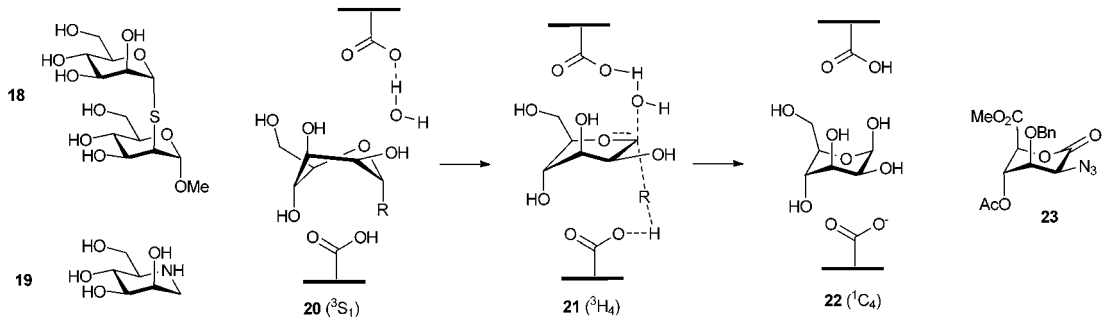
Next to these two-step probes, direct probes based on fluoroglycosides have been reported. These probes have the visualization moiety already installed on the carbohydrate ring, allowing for the direct visualization of the trapped enzymes. In this way, endo-β-xylanase and cellulase enzymes were labeled with xylobioside probes **26**, and the kinetic parameters for inhibition were similar for the tagged and untagged probes.[33] The probes were used to label both pure enzyme samples and the secreted proteome of the soil bacterium *Cellulomonas fimi*.

A beneficial effect of the lipophilic BODIPY moiety on enzyme binding kinetics was observed with probes developed to label acid β-glucocerebrosidase (GBA), a retaining exoglucosidase, which degrades glucosylceramide (Fig. 4). 2-Deoxy-2-fluoroglucosides with different anomeric leaving groups (**27**) and cyclophellitol-based probes (**28**) were compared for their activity-based inhibition properties of GBA, revealing that the fluoroglucoside probes were much less potent inhibitors than the cyclophellitol-based probes.[34] Several factors may contribute to this remarkable difference in inhibition properties and labeling affinity. The 2-deoxy-2-fluoroglycosides were designed to decrease the reactivity of the donor glycoside through depletion of electron density at the anomeric carbon, leading to stabilization of the covalent glycosyl–enzyme adduct. As said,

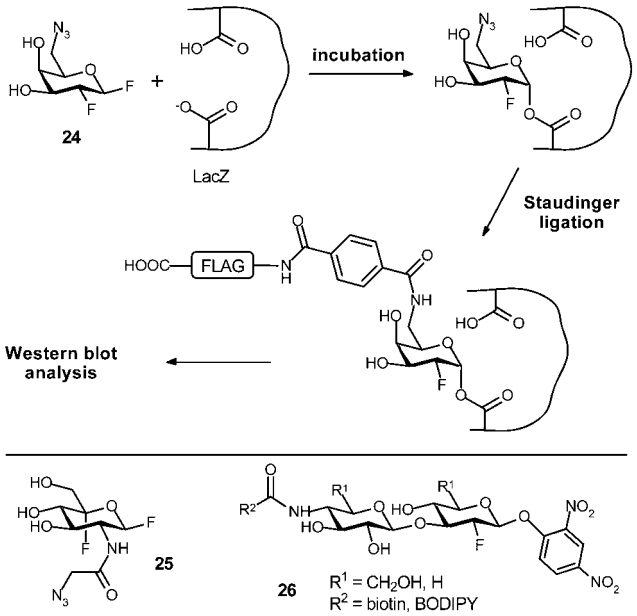


15 16 17

Fig. 3 Reactivity of C2/C5-fluoro glycoside inhibitors.



Scheme 3 Probes for α-mannosidases, and the catalytic itinerary ($^3S_1 \rightarrow {}^3H_4 \rightarrow {}^1C_4$), and lactone **23**.



Scheme 4 Two-step and direct probes based on fluoroglycosides **24–26**.

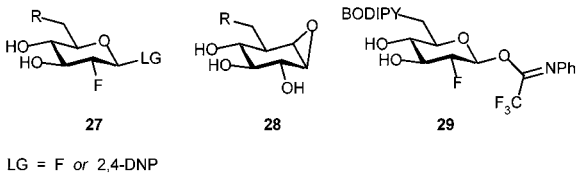


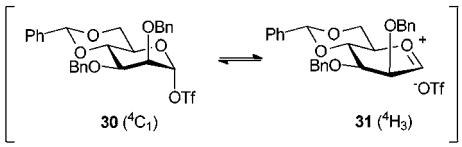
Fig. 4 GBA probes (R = azide, BODIPY).

this reduced reactivity is already embedded in the glycosyl fluoride or (di)nitrophenyl glycoside. In contrast, the cyclitol epoxide inhibitor is optimally geared to *enhance* initial reaction within the glycosidase active site: it should be more electron-rich, and the epoxide is optimally positioned for protonation by the general acid/base catalyst. Only after activation and substitution by the catalytic nucleophile is an intermediate formed that is comparatively more stable than a normal glycosyl–enzyme adduct due to a relatively stable ester linkage (compared to the natural acylal intermediate). Returning to the 2-deoxy-2-fluoroglycosides: here the intrinsic decrease in reactivity was compensated by introducing a potent leaving group, and in the case of the anomeric fluoride series, the propensity of the fluorine to depart within a glycosidase active site to become substituted by the nucleophile appeared such that also mutant enzymes (lacking the acid/base catalyst) were effectively modified. Indeed, the anomeric fluorine does not require, and likely neither invites protonation, in other words, does not capitalize on the intrinsic mechanism of a retaining glycosidase. In line with this reasoning, we found that 2-deoxy-2-fluoroglucoside probe **29**, bearing a β-*N*-phenyl-trifluoroacetimidate as anomeric leaving group as developed by Yu and Sun for glycosylation chemistry,[35] inhibited and labeled GBA much more potently than the corresponding compound equipped with an anomeric fluoride.[36] In contrast to the anomeric fluoride, this leaving group required enzymatic protonation in the active site in order to be expelled, since mutant GBA lacking the acid/base residue proved inert towards imidate probe **29** featuring the anomeric acetimidate, but not the analogous anomeric fluoride probe. In truth, acetimidate probe **29** still compares poorly with cyclitol epoxide-based probe **28**, which may have to do with the lack of a hydroxyl substituent at C-2. Grafting a fluorine atom at C-5 in an otherwise fully glucopyranose-configured compound may therefore lead to even more potent 'Withers-type' glycosidase activity-based probes.

Glycochemistry

In the previous section details and merits of covalent glycosyl–enzyme adducts in the enzymatic hydrolysis of glycosidic bonds are discussed. In recent years the importance of covalent intermediates in chemical glycosylation reactions has also taken up a prominent position. In this section, some studies pertaining to the formation and relevance of covalent intermediates, featuring components of the activating agent covalently attached to the donor glycoside, in chemical glycosylation pathways are discussed.

A breakthrough in our understanding of reactive covalent intermediates involved in glycosylation reactions came with the first observation made by Crich and Sun of a covalent mannosyl triflate (**30**, Scheme 5).[37] Serendipitous pre-activation of a 4,6-*O*-benzylidene-protected sulfoxide donor prior to addition of the nucleophile provided the β-linked disaccharide product with



Scheme 5 Intermediates upon pre-activation of 4,6-*O*-benzylidene protected mannose.

unexpectedly high stereoselectivity. This prompted the investigation of the intermediate formed upon pre-activation, and using low-temperature NMR spectroscopy, the anomeric α-triflate **30** was identified. The existence of this species suggested that the high β-selectivity observed arose from the S_N2-like substitution on the axial α-triflate. When this covalent intermediate dissociates to the (solvent-separated) ion pair, the mannosyl oxocarbenium ion takes up a $^4H_3$ half-chair conformation (**31**) (or closely related $B_{2,5}$ boat conformation), providing the α-product upon nucleophilic attack.[38] In this scenario, the equilibrium between the covalent intermediate and the (solvent-separated) oxocarbenium ion, in combination with the rate of substitution on both species, determines the stereoselectivity of the reaction. The benzylidene group in **30** serves to stabilize the anomeric triflate with respect to the oxocarbenium ion **31** by conformationally restricting the mannosyl chair structure, and hampering the flattening of the ring to accommodate the sp^2-hybridized oxocarbenium ion. In addition, the benzylidene ring locks the C-6 oxygen atom in the most electron-withdrawing *tg* conformation,[39] thereby electronically disfavoring the formation of the anomeric cation. In a recent study, primary ^{13}C kinetic isotope effects (KIEs) were used to dissect the mechanisms underlying the formation of the α- and β-mannosyl products in the benzylidene mannosyl system.[40] It was revealed that the formation of the β-products occurred *via* an associative mechanism, where the formation of the α-products proceeded through a dissociative pathway.

Besides conformational restriction, the stabilization of anomeric triflates has also been attained through the incorporation of electron-withdrawing substituents.[41,42] For example, using a series of increasingly fluorinated mannopyranosides (**32–34**, Fig. 5), it was established that the stability of the intermediate triflate increased upon degree of fluorination.[41]

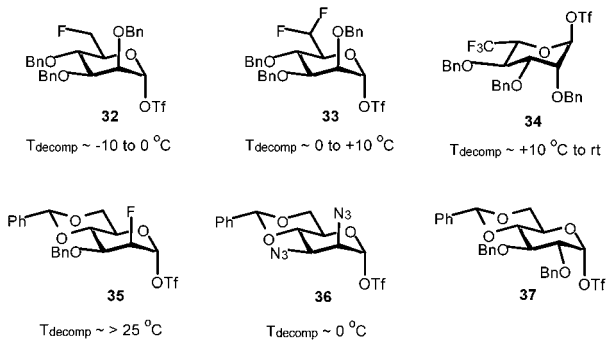
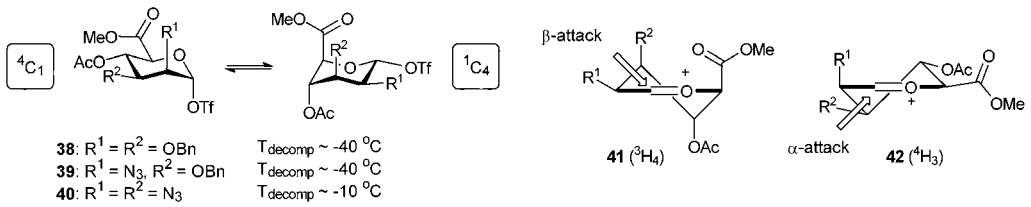


Fig. 5 Triflates **32–37**.

And in this case, the stability of the anomeric triflates was mirrored in the stereoselectivity of the mannosylation reactions: the more stable triflate gave the highest β-selectivity. It should be noted however, that the stability of an anomeric triflate is no general measure for the amount of S_N2-like substitution, and consequently the stereoselectivity of a glycosylation reaction. This is illustrated by the benzylidene-protected mannosyl triflates (**35** and **36**, Fig. 5), of which 2-fluoromannosyl triflate **35**[43] and 2,3-diazidomannosyl triflate **36**[44] are both more stable than mannosyl triflate **30**,[37] while condensation reactions with triflates **35** and **36** proceed with a significantly diminished β-selectivity. In fact, in many cases (if not most), the observation of a single anomeric triflate does not guarantee an S_N2-like pathway. For example, benzylidene-protected glucosyl donors can be activated to provide an α-triflate intermediate (such as **37**, Fig. 5), but these are substituted in the ensuing condensation event with retention of configuration at the anomeric center to provide α-glucosides with good selectivity. This stereochemical outcome can be rationalized by assuming that the observed anomeric triflate serves as a reservoir for the more reactive oxocarbenium ion, which reacts in an α-selective manner. Alternatively, it can be hypothesized that the axial α-triflate is in dynamic equilibrium with the more reactive equatorial β-triflate, which can be substituted in an S_N2-like manner to provide the α-linked products, in line with Lemieux's *in situ* anomerization protocol featuring anomeric halides.[45] Obviously, axial α-triflates benefit from a strong stabilizing anomeric effect, making these species largely favored over their equatorially linked counterparts. As a consequence, a large number of axial α-triflates have been reported[46] while there are only very few reports on equatorial triflates,[47] of which the best studied examples are the mannuronic acid triflates described below. As for the benzylidene glucosyl triflates, primary ^{13}C KIEs have indicated that both the α- and β-products in glycosylation of 4,6-*O*-benzylidene glucosyl donors are formed in an S_N2-like fashion, implicating the equatorial β-glucosyl triflate as a product-forming intermediate.[40]

Condensation reactions involving mannuronic acid donors proceed with high stereoselectivity to provide β-linked products. This selectivity can be explained by invoking an S_N2-like displacement mechanism on an anomeric α-triflate. Indeed, these intermediates have been observed by NMR spectroscopy (Scheme 6). However, the triflates obtained by pre-activation of the corresponding donors occurred as mixtures of two conformers, a $^4C_1$ chair conformer with an axial triflate, and a $^1C_4$ conformer placing the triflate in an equatorial direction. Not only does the latter triflate lack the anomeric stabilization present in its $^4C_1$ chair counterpart, it also places three substituents in sterically unfavorable axial positions. Presumably these triflates adopt this unexpected conformation because of the electron-depleted anomeric center. To stabilize the partial positive charge at the anomeric center, the mannuronic acid adopts a conformation approaching the $^3H_4$ half-chair conformation (**41**, Scheme 6), which represents the most favorable conformation for the mannuronate oxocarbenium ion.[48–51] Notably, all mannuronic acid triflates observed to date are significantly more labile than what would be expected based



Scheme 6 Conformational mixture of mannuronic acid triflates **38–40**, and the corresponding oxacarbenium ion half chairs **41**/**42**.

on the consideration that the electron-withdrawing carboxylic acid ester at C-5 should disfavor collapse of the triflate into the corresponding oxocarbenium ion (**41**/**42**, Scheme 6). Taken together, another pathway to account for the high β-selectivity of the mannuronic acid donors can be envisaged, in which a $^3H_4$ half-chair oxocarbenium ion-like intermediate is selectively attacked on the diastereotopic face that leads to the formation of the chair product, that is, the β-face (Scheme 6).

Because all common glycosylation conditions involve the use of electrophilic activators having triflate counter ions (Ph_2SO/Tf_2O, AgOTf/*p*-TolSCl, NIS/TfOH for thioglycosides, TMSOTf, TfOH for glycosyl imidates), anomeric triflates can be postulated to be an intermediate in the vast majority of glycosylation reactions performed to date. Whether they are actually the glycosylating species or merely a resting state depends on many variables, including the reactivity of the coupling partners, reaction temperature, solvent and concentration.

Besides anomeric triflates, various other covalent species have been produced upon glycosyl donor activation. For example, Gin and Garcia described that the intermediate formed in their dehydrative glycosylation protocol is an oxosulfonium triflate species.[52] These species are more stable than the corresponding anomeric triflates, and it has been shown that per-*O*-methyl mannosyl triflate is rapidly converted into oxosulfonium triflate **43** by treatment with diphenyl sulfoxide (Fig. 6). The higher stability of oxosulfonium triflates with respect to covalent triflates was used in the study of covalent intermediates formed upon activation of sialic acids.[53] While pre-activation of sialic acid thio-donors with a stoichiometric amount of a thiophilic promoter resulted in rapid elimination of the putative anomeric triflate, the addition of an excess of diphenyl sulfoxide yielded a diastereomeric mixture of oxosulfonium triflates (**44**, Fig. 6). Through this stabilized intermediate,[54] a variety of acceptors were glycosylated with moderate α-stereoselectivity. The stereoselectivity in these condensations was improved by conducting the reaction in a mixture of acetonitrile–dichloromethane. This solvent mixture is commonly used for the stereoselective construction of α-sialosides, and indicates that the product-forming species in these condensation reactions is not the observed oxosulfonium triflate, but rather that a solvent-stabilized oxocarbenium ion-like species, is at the basis of the observed selectivity.

The addition of diorganosulfides to anomeric triflate intermediates leads to the formation of glycosyl sulfonium ions (such as **45** and **46**, Fig. 6), which can be rather stable, and in cases even be used as storable glycosyl donors.[55,56] Notably, most glycosyl sulfonium ions prefer to place the anomeric sulfonium ion moiety in an equatorial position. In some cases, these glycosyl sulfonium ions can be used for the stereoselective formation of glycosidic bonds, through the direct S_N2-like displacement of the intermediates. The reactivity and selectivity of these species critically depend on both the substituents of the glycosyl core as well as the substituents on the sulfonium center. Boons and co-workers elegantly exploited the stability of the intramolecular sulfonium ions for the stereoselective construction of α-glucosidic and α-galactosidic bonds.[57] Sulfonium species **47** (Fig. 6) can be obtained using a chiral SPh auxiliary appended at the C-2 position, or through aromatic substitution by an oxothiane intermediate.[58] This *trans*-decalin sulfonium system is relatively stable and can be substituted in

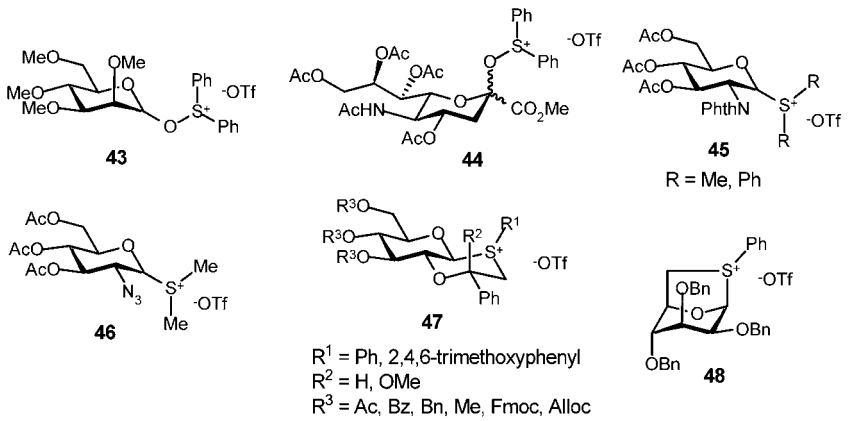


Fig. 6 Various covalent intermediates.

an S_N2-like manner from the α-face to provide the 1,2-*cis*-linked target products. Also in this system the protecting groups on the carbohydrate core played an important role, and it was shown that electron-withdrawing protecting groups promoted an S_N2-like reaction pathway over the alternative S_N1 trajectory by disfavoring collapse of the "covalent" sulfonium ion into the oxocarbenium ion.[59] Bicyclic mannosyl sulfonium ions, such as **48** (Fig. 6), have also been generated, and these proved to be stable at room temperature for several hours.[60] Nucleophilic substitution of these species mainly produced the β-configured product. Since substitution of **48** in an S_N2-like manner would give the α-product, a $^3H_4$ half-chair oxocarbenium ion (preferred for mannosides) was invoked as product-forming intermediate.

Conclusions

In summary, the expanding body of studies on stability and reactivity of donor glycosides in glycochemistry and glycobiology has witnessed a remarkable increase in examples of intermediates in which the substrate/donor glycoside after activation is captured as a covalent intermediate prior to further processing towards the product. Detailed analysis of such intermediates in chemical carbohydrate synthesis has aided in our understanding of pathways and mechanisms involved in stereoselective glycosylation events, and whether or not covalent intermediates are actually involved in a glycosylation, or merely serve as thermodynamic sinks to store reactive species. Likewise, tailored glycosidase probes have unambiguously established the existence of covalent enzyme–substrate intermediates in the case of retaining glycosidases, and although it is tempting to assume that these intermediates are crucial in the process towards glycoconjugate hydrolysis, also here it is not excluded that the actual species that will capture water in the enzyme active site is in fact a charge-separated glycoside species. From a practical point of view, modified carbohydrate donors are finding increasing application in chemical glycobiology studies. New generations of 'Withers-type' 2-deoxy-2-fluoroglycosides emerge that, next to their application in structural biology studies on isolated enzymes, are potent and selective enough to also allow activity-based profiling of retaining glycosidases in complex biological samples, a promising yet underdeveloped field of research in chemical biology, in comparison with esterase/protease activity-based profiling. We envisage that the translation of chemical glycosylation methodology to a glycobiology setting by the use of anomeric leaving groups that are commonly activated by Brønsted acids, such as anomeric phosphates, phosphoramidates and phosphoramidites and 3-methoxy-2-pyridyl (MOP) glycosides[24b] will yield improved glycosidase probes. Also, tailored and shelf-stable donor glycosides equipped with a good anomeric leaving group have found their use in chemoenzymatic synthesis of glycoconjugates, for instance involving a transglycosylase reaction effected by a mutant glycosidase lacking the general acid/base or nucleophilic residue.[61] Interestingly, the first examples of shelf-stable donor glycosides, that can be made to react in a chemical glycosylation reaction upon transfer to the acceptor, have appeared in literature as well.

As is described here, mechanism-based glycosidase inhibitors have been used to study reaction pathways by which glycosidases cleave glycosidic linkages and have recently served as a starting point for the development of activity-based glycosidase probes. Very recently we were able to expand this concept by turning a cyclitol aziridine into a broad-spectrum, retaining glucosidase probe, thereby demonstrating the potency of activity-based glycosidase profiling as a discovery tool to unearth new glycosidase activities and to classify these enzymes in various categories.[62] It is our expectation that activity-based probes targeting distinct glycosidase families will become available in the near future for chemical glycobiology studies. In contrast, however, related probes for the study of glycosyl transferases have not emerged yet, and this situation is perhaps rooted in the fact that – compared to glycosidases – effective glycosyl transferase inhibitors that can serve as a starting point are scarce. Yet, several retaining glycosyl transferases that make use of Leloir donors are found to have a carboxylate residue in their active site situated at a position suggesting their mechanistic involvement as a nucleophile in the displacement of the XDP leaving group to form an acylal intermediate rather like what is observed in the hydrolysis of a glycosidic bond as effected by retaining glycosidases.[63] In fact the mechanistic aspects of retaining glycosyl transferases have been subject of considerable studies and very recently Davis and co-workers reported on an S_Ni-type mechanism, in which the XDP leaving group leaves the enzyme active site in a such a way that the incoming nucleophile (the acceptor glycoside) is allowed to approach the developing oxocarbenium ion from the same side, resulting in net retention of configuration.[64] Future research will demonstrate whether this is a general mechanism or that, perhaps more likely, more mechanisms can be invoked depending on the nature of the glycosyl transferase at hand. One way to demonstrate the involvement of covalent intermediates would be the design of an activity-based probe based on the appropriate XDP-glycoside.

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